PI 1071440

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

September 27, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/403,893*
FILING DATE: *August 16, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US03/25681*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

M. Sias

M. SIAS
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

08-19-02 ~~A~~ IPR

PTO/SB/16 (10-01)
Approved for use through10/31/2002 OMB 0651-0032
U.S Patent and Trademark Office, U S DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ET730024659US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Alan D.	Barrett	Galveston, Texas
David	Beasley	Galveston, Texas
Michael	Holbrook	Oklahoma City, Oklahoma

☐ *Additional inventors are being named on th____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

DIAGNOSIS OF FLAVIVIRUS INFECTIONS USING ENVELOPE PROTEIN DOMAIN III

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number [ ] → *Type Customer Number here*

OR

Place Customer Number Bar Code Label here

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 11 ☐ CD(s), Number

☐ Drawing(s) *Number of Sheets* ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number 50-0772

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($) $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S Government agency and the Government contract number are: U.S. Department of Health and Human Services Centers for Disease Control Contract No. U90/CCU618754-01

Respectfully submitted,

SIGNATURE [signature]

Date 08-16-2002

TYPED or PRINTED NAME Peter Rogalskyj

REGISTRATION NO. *(if appropriate)* 38,601

Docket Number: 026.00720 (BARR-AD-02B)

TELEPHONE 585-393-3001

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C.

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant(s): Barrett et al.			Docket No. 026.00720 (BARR-AD-02B)
Serial No. To Be Assigned	Filing Date Herewith	Examiner Not Assigned	Group Art Unit Not Assigned

Invention: **DIAGNOSIS OF FLAVIVIRUS INFECTIONS USING ENVELOPE PROTEIN DOMAIN III**

I hereby certify that the following correspondence:

Provisional Application for Patent Cover Sheet (1 page) (in duplicate) and check for $80.00

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August 16, 2002
(Date)

Peter Rogalskyj
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("Express Mail" Mailing Label Number)

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POBA/REV02

EL940389013 US 081602

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant: Barrett et al.			Docket No. 026.00720 (BARR-AD-02B)
Serial No. To Be Assigned	Filing Date Herewith	Examiner Not Assigned	Group Art Unit Not Assigned

Invention: DIAGNOSIS OF FLAVIVIRUS INFECTIONS USING ENVELOPE PROTEIN DOMAIN III

I hereby certify that the following correspondence:

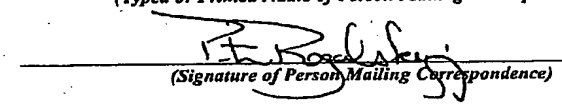
Provisional Patent Application (11 pages)

(Identify type of correspondence)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231 on

August 16, 2002
(Date)

Peter Rogalskyj
(Typed or Printed Name of Person Mailing Correspondence)



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P06A/REV02

TITLE: DIAGNOSIS OF FLAVIVIRUS INFECTIONS USING ENVELOPE PROTEIN DOMAIN III

INVENTORS: Alan D. Barrett
David Beasley
Michael Holbrook

DOCKET NO.: 026.00720 (BARR-AD-02B)

I:\026\00720\5

DIAGNOSIS OF FLAVIVIRUS INFECTIONS USING ENVELOPE PROTEIN DOMAIN III

The present invention was made with the support of the U.S. Department of Health and Human Services Centers for Disease Control Contract No. U90/CCU618754-01. The Federal Government may have certain rights in this invention.

There are 100 million dengue infections each year. There is a worldwide market for dengue diagnostics to identify which dengue virus is a primary infection and distinguishing primary from secondary infections. There is a need to differentiate human West Nile and St. Louis encephalitis infections in the USA. There is a need for more specific equine diagnostics for West Nile in the USA. Due to many arboviral infections having similar clinical signs at the beginning of infections, diagnostics that distinguish between viruses would be very useful. Discussion at a recent West Nile symposium organized by the CDC focused on the need for antigen for diagnostic assays and the requirement for improved specificity of assays to allow the discrimination of West Nile from St. Louis encephalitis and other flavivirus infections. Current antibody-based diagnostics for West Nile in the United States are primarily utilizing whole virus antigen prepared by the CDC.

The flaviviruses are small spherical enveloped viruses that have three structural proteins: core, membrane, and envelope (E). The crystallographic

structure of the ectodomain of Central European tick-borne encephalitis (TBE) virus has been solved (Rey et al. Nature, 1995) and found to consist of three domains (I, II, and III). Domain III is approximately 100 amino acids in length (approximately 10kDa is size). The E protein is approximately 500 amino acids in length, and domain III is approximately amino acids 300-400, depending on the flavivirus. Recombinant Domain III can be readily expressed in a recombinant system, and the resultant protein is extremely stable largely due to the presence of a single disulphide bridge. U.S. Patent No. 5,895,651 to Simmons et al. ("Simmons") describes the use of recombinant domain III (termed domain B in the patent due to old nomenclature) derived from dengue virus strains and expressed as a fusion with maltose binding protein ("MBP") as a candidate vaccine immunogen due to its ability to induce neutralizing antibodies. This work is based on immunization of mice with MBP fusion protein of dengue virus domain III that induces protective immunity. Simmons relates to DEN1-4 only and also the DENX-MBP fusion protein.

In the present work, we express domain III as a fusion protein, the fusion partner (e.g. MBP) is cleaved, and domain III is purified.

Our laboratory has been undertaking studies on investigating the interaction of the flavivirus receptor-binding domain (i.e., domain III) with cell receptors. As part of these studies, we have been studying the reactivity of monoclonal antibodies with domain III. During these studies, we found that domain III was a very specific antigen in ELISAs compared to whole virus antigen. The specificity of domain III recognition by

antibodies has important implications for flavivirus diagnostics because flaviviruses share so many E protein epitopes that virus-specific diagnosis is very difficult. Examples of this are the difficulty in distinguishing West Nile from St. Louis encephalitis and the difficulty in distinguishing the four dengue viruses from each other, and primary from secondary dengue infection.

We propose using purified domain III (i.e., domain III which is not part of a fusion protein) as a diagnostic reagent for flavivirus infections. Ease of expression and purification of recombinant domain III could make it a cheap and effective reagent for diagnostics. In addition, domain III seems to encode type-specific epitopes for flaviviruses. Therefore, domain III could be used to generate antibodies, such as monoclonal antibodies, or antibody fragments that could be used to detect a particular virus specifically.

As used herein, "antibodies" are meant to include polyclonal antibodies and monoclonal antibodies capable of binding to purified domain III, as well as fragments of these antibodies, and humanized forms. Humanized forms of the antibodies of the subject invention may be generated using one of the procedures known in the art such as chimerization. Fragments of the antibodies of the present invention include, but are not limited to, the Fab, the $F(ab')_2$, and the Fc fragments. Suitable antibodies or fragments thereof include those which are specific for purified domain III. Hybridomas which are capable of producing the above-described antibodies are also contemplated. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (see Campbell, A.M., Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers, Amsterdam, The Netherlands (1984) and St. Groth, et al., J Immunol Methods 35:1-21 (1980) ("Campbell")). Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be immunized with the antigenic purified domain III (or an antigenic fragment thereof). Methods for immunization are well known in the art. Such methods include subcutaneous or intraperitoneal injection of the polypeptide. One skilled in the art will recognize that the amount of the domain III antigen used for immunization will vary based on the animal which is immunized, the antigenicity of the domain III antigen, and the site of injection.

The domain III antigen which is used as an immunogen may be modified or administered in an adjuvant in order to increase the domain III antigen's antigenicity. Methods of increasing the antigenicity of an antigen are well known in the art and include, but are not limited to, coupling the antigen with a heterologous protein (such as a globulin or beta-galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/O-Ag 15 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art can be used to identify the hybridoma cell which

produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz, et al., Exp Cell Res 175:109-124 (1988)).

Hybridomas secreting the desired antibodies are cloned and the class and subclass are determined using procedures known in the art (see, e.g., Campbell).

For polyclonal antibodies, antibody containing antisera is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

In accordance with the above discussion, we also contemplate methods of producing an antibody specific for a polypeptide of the present invention in a host. The method comprises selecting the isolated domain III antigen or an antigenic portion thereof and introducing the selected domain III antigen or antigenic portion thereof into a host to induce production of an antibody specific for domain III in the host.

The present invention also relates to the above-described antibodies in detectably labeled form. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc.), fluorescent labels (such as FITC or rhodamine, etc.), paramagnetic atoms, etc. Procedures for accomplishing such labeling are well known in the art (for example see Sternberger, L.A., et al., J Histochem Cytochem 18:315 (1970); Bayer, E.A., et al., Meth Enzym 62:308 (1979); Engval, E., et al., Immunol 109:129 (1972); and Goding, J.W., J Immunol Meth 13:215 (1976)).

The antibodies (labeled or otherwise) or fragments thereof described above can be used for in vitro, in vivo, and in situ assays to screen for the presence or absence of various flaviviruses and/or flavivirus infections (e.g., dengue, West Nile, St. Louis encephalitis, tick-borne encephalitis, yellow fever, Japanese encephalitis). Illustratively, the cleaved, purified domain III antigens and anti-domain III monoclonal antibodies or polyclonal antisera can be used in the specific diagnosis of flavivirus infections (for instance, the discrimination of JE serocomplex virus infections such as West Nile and St Louis encephalitis). As one skilled in the art will recognize, cleaved, purified domain III antigens can be used, for example, to ascertain the presence or absence of antibodies to a particular flavivirus in a physiological sample taken from a subject (e.g., a human or other animal) to determine whether such subject has been previously exposed to the particular flavivirus. Additionally or alternatively, the cleaved, purified domain III antigens can be used to raise antibodies, and the antibodies, in turn, can be used to determine whether a particular flavivirus is present in a sample.

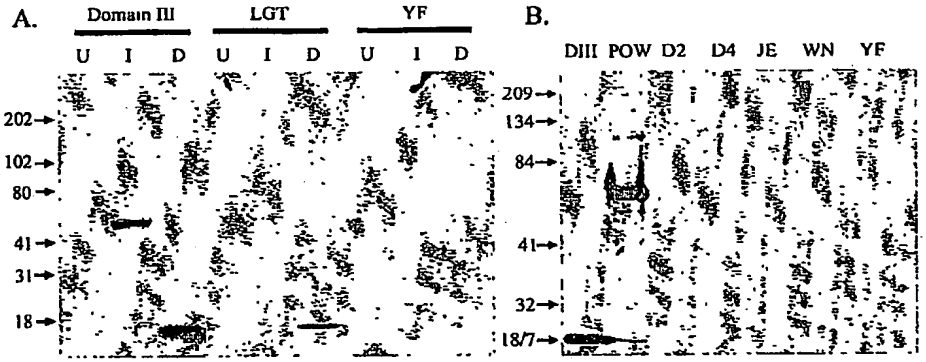
The methods described above can be used in conjunction with dipstick technologies or with any other technique for contacting the above-described domain III antigen or antibodies with a physiological sample or other sample.

Illustratively, we have used E. coli to express domain III of different flaviviruses (Langat, Powassan, Omsk hemorrhagic fever, Kyasanur Forest disease, West Nile, dengue-4, Central European tick-borne encephalitis,

and yellow fever). Domain III is approximately 100 amino acids in length (approximately 10kDa in size). The E protein is approximately 500 amino acids in length and domain III is approximately amino acids 300-400, depending on the flavivirus. Domain III can be amplified by RT-PCR and cloned into expression plasmids. We have used GST, his-tag and MBP fusion proteins. The best results have been obtained with MBP constructs. Rabbit antisera have been prepared to Langat, West Nile and dengue-4 recombinant domain IIIs. In Western blots, the rabbit antisera show specificity for domain III. For example, the Western blot set forth in Figure 1 shows specificity of Langat virus domain III rabbit antisera. Note that domain III has a molecular weight of approximately 10kDa while the envelope(E) protein is approximately 53kDa.

FIGURE 1

Specificity of Langat virus domain III. Panel A: Recognition of domain III by Langat antisera; U= uninfected Vero cells, I= virus-infected Vero cells, D=E.coli expressed recombinant Langat virus domain III probed with rabbit anti-Langat virus domain III (Domain III), Langat virus mouse immune ascitic fluid (LGT) or yellow fever virus mouse immune ascitic fluid (YF). Panel B: Reactivity of rabbit anti-Langat virus domain III serum with Langat virus domain III or Vero cells infected with Powassan, dengue-2, dengue-4, Japanese encephalitis, West Nile or yellow fever virus.

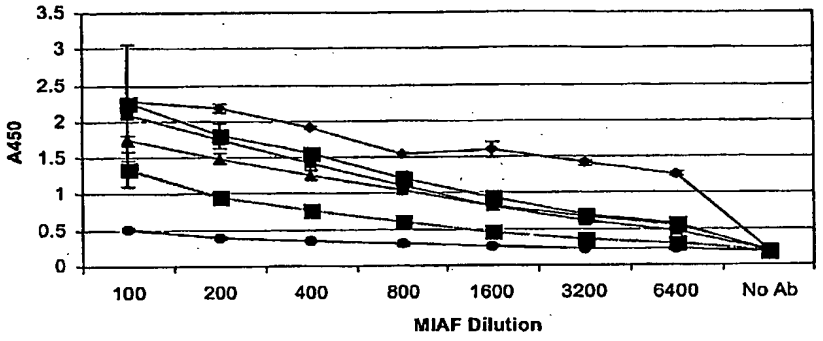


The specificity of reactivity was confirmed in an ELISA format (see Figure 2). West Nile whole virus antigen was compared to West Nile virus recombinant domain III against a variety of mouse immune ascitic fluids ("MIAF"). As can be seen in Panel A, the West Nile MIAF shows the best reaction with the West Nile whole virus antigen, but there is extensive cross-reaction with MIAFs raised against closely related viruses (Murray Valley encephalitis [MVE], St. Louis encephalitis [SLE], and Japanese encephalitis [JE]). Panel B, shows the results of using West Nile recombinant

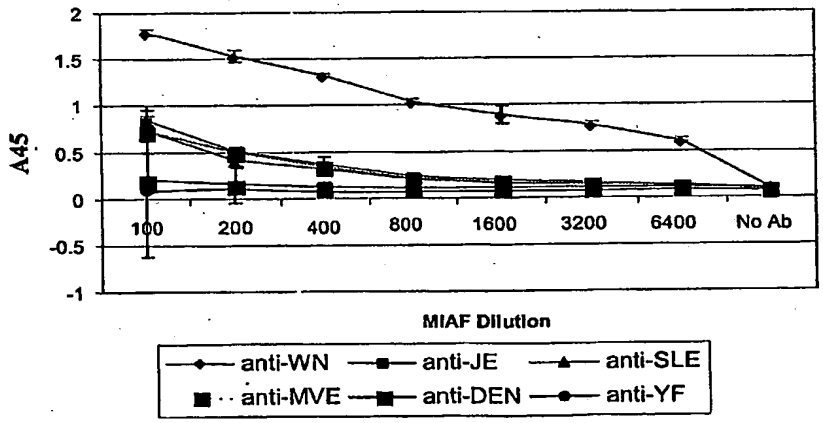
domain III as antigen. The ELISA shows a strong specificity for West Nile while retaining a high signal compared to the background and other MIAFs. One weakness/unknown is that the ELISA has not been used with human sera but MIAFs contain antibodies that recognize many flavivirus cross-reactive epitopes as shown by the results in Panel A. Similar specificity is seen in indirect immunofluoresence tests (data not shown).

FIGURE 2

(a) WN NY99 Ag coated



(b) WN NY99 rEIII Ag coated



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As shown by the US Army/Navy, using antisera prepared against MBP-dengue domain III fusion proteins, our recombinant domain III rabbit antisera shows specificity in neutralization tests (Table 1).

Table 1: Cross-neutralization tests using rabbit antisera prepared against LGT and WN domain IIIs

	80% PRNT		50% PRNT	
Virus	anti LGT	anti-WN	anti LGT	anti-WN
DEN2	<20	<20	<20	<20
DEN4	nt	nt	nt	nt
JE	<20	<20	<20	<20
WN	<20	320	<20	>320
YF	<20	<20	<20	<20
LGT	40	<20	80	<20
POW	<20	<20	20	<20

nt: Not tested

Although preferred embodiments of the present invention are depicted and described in detail hereinabove, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of these illustrative embodiments, and these modifications, additions, substitutions and the like are, therefore, considered to be within the scope of the present invention.

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PI 1071441

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

September 30, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/445,581*
FILING DATE: *February 06, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US03/25681*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

M. Sias

M. SIAS
Certifying Officer

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02/06/03 J1032 U.S. PTO

02-10-03 60445581 US060

60445581 02/06/03 PTO

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (c)

Docket Number	UTSG:260USP1	Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Barrett	Alan		Galveston, Texas
Beasley	David		Galveston, Texas
Holbrook	Michael		Oklahoma City, Oklahoma

TITLE OF THE INVENTION (280 characters max)

COMPOSITIONS AND METHODS RELATED TO FLAVIVIRUS ENVELOPE PROTEIN DOMAIN III ANTIGENS

CORRESPONDENCE ADDRESS

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
USA

ENCLOSED APPLICATION PARTS (*check all that apply*)

- ☒ Specification — *Number of Pages* 97
- ☒ Drawing(s) — *Number of Sheets* 9
- ☐ Small Entity Statement
- ☒ Other (specify) Statement as Required Under 37 CFR 1.821(f); Paper Copy and CD Rom of Sequence Listing.

METHOD OF PAYMENT (*check one*)

- ☒ A check or money order is enclosed to cover the Provisional filing fees.
- ☒ The Assistant Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: 50-1212/UTSG:260USP1/CPL should any fees be missing or deficient.
- ☐ Pursuant to 37 CFR 1.53(g) this provisional application is being filed without a filing fee. Please send the "Notice to File Missing Parts" form pursuant to 37 CFR 1.53(g).

PROVISIONAL FILING FEE AMOUNT $80

☒ Applicant is entitled to Small Entity Status Pursuant to 37 C.F.R. § 1.27.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

- ☐ No.
- ☒ Yes, the name of the U.S. Government agency and the Government contract number are: U90/CCU618754-01 US DEPARTMENT OF HEALTH AND HUMAN SERVICES CENTERS FOR DISEASE CONTROL.

CERTIFICATE OF EXPRESS MAILING
NUMBER EV 119101539 US
DATE OF DEPOSIT 02/06/03

Respectfully submitted,

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Date: February 6, 2003

EXPRESS MAIL MAILING LABEL	
NUMBER	EV 119101539 US
DATE OF DEPOSIT	February 6, 2003

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
ALAN BARRETT; DAVID BEASLEY
AND MICHAEL HOLBROOK

Serial No.: UNKNOWN

Filed: FILED CONCURRENTLY HEREWITH

For: COMPOSITIONS AND METHODS RELATED TO FLAVIVIRUS ENVELOPE PROTEIN DOMAIN III ANTIGENS

Group Art Unit: UNKNOWN

Examiner: UNKNOWN

Atty. Dkt. No.: UTSG:260USP1

STATEMENT AS REQUIRED UNDER 37 C.F.R. § 1.821(f)

BOX SEQUENCE
Commissioner for Patents
Washington, D.C. 20231

Commissioner:

Submitted herewith is a computer readable form and a paper copy of the sequence listing of those sequences in the captioned patent application. The computer readable form of the sequence listing is the same as the paper copy of the sequence listing. The sequence information provided in the Specification is also the same as the sequence listing of the enclosed computer readable and paper forms of the sequence listing.

Respectfully submitted,

Charles P. Landrum

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Date: February 6, 2003

PATENT

UTSG:260USP1

PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT

for

COMPOSITIONS AND METHODS RELATED TO FLAVIVIRUS ENVELOPE PROTEIN DOMAIN III ANTIGENS

by

Alan Barrett

David Beasley

and

Michael Holbrook

EXPRESS MAIL MAILING LABEL	
NUMBER	EV 119101539 US
DATE OF DEPOSIT	February 6, 2003

BACKGROUND OF THE INVENTION

The government may own rights in the present invention pursuant to contract number U90/CCU618754-01 from U.S. Department of Health and Human Services Centers for Disease Control.

1. Field of the Invention

The present invention relates generally to the fields of virology, immunology and diagnostics. More particularly, it concerns antibodies directed to and antigens derived from West Nile virus E protein domain III in compositions and methods for detection of West Nile virus.

2. Description of Related Art

West Nile virus (WN) is a member of the Japanese encephalitis (JE) serocomplex of the genus *Flavivirus* (Family *Flaviviridae*). This virus was first isolated from a febrile woman in the West Nile province of Uganda in 1937, and now has an almost worldwide distribution including parts of Africa, Asia, Europe and, most recently, North America. Kunjin virus, now re-classified as a subtype of West Nile virus, is found in Australasia.

Since 1999, the United States has experienced annual epidemics of WN disease in humans and animals over an expanding geographical range. WN virus has been isolated in 42 states, and more than 2,100 cases of human disease resulting in 95 deaths had been reported during the summer of 2002 (MMWR , 2002a). Several of these cases are suspected to have originated from virus transmitted during blood transfusion and/or organ transplantation (MMWR, 2002b). Outbreaks of WN disease with neurological manifestations have also been reported in Eastern Europe, North Africa and Israel since the mid-1990s (reviewed by Murgue *et al.*, 2002).

Other members of the JE serocomplex include JE virus, found throughout Asia, St. Louis encephalitis (SLE) virus, found in the Americas, and Murray Valley encephalitis (MVE) virus, found in Australia and New Guinea. These viruses are antigenically similar to WN virus, and their co-circulation in several regions of the world has complicated the specific diagnosis of infections by these viruses in humans and other

hosts (Fonseca *et al.*, 1991; Martin *et al.*, 2002). Current protocols for the serological diagnosis of WN virus infection in the United States rely primarily on preliminary screening for WN virus-reactive IgM/IgG antibody by capture ELISA and confirmation by plaque reduction neutralization test (PRNT) (CDC, 2001), a process which results in considerable delays in the reliable reporting of accurate case numbers, and requires the confirmatory testing to be performed in specialized laboratories.

WN is clearly an emerging and significant public health problem. There is a need to limit the impact of WN virus including the development of more specific diagnostic assays for patients, animal and insect vectors, and blood/organ donor screening (Petersen *et al.*, 2002).

SUMMARY OF THE INVENTION

The present invention takes advantage of the observation that a West Nile virus E protein domain III (WN-EIII) antigen can be used to specifically detect West Nile virus and antibodies against West Nile virus. Various embodiments of the invention are directed to compositions and methods related to detecting West Nile virus in a patient, animal, biological or other types of samples.

The present invention includes compositions and methods for the detection or diagnosis of flavivirus or West Nile virus. Recombinant West Nile virus E protein domain III (WN-rEIII) can be expressed in *E. coli* as a fusion protein to produce a soluble protein that can be purified. Rabbit antisera raised against WN-rEIII shows virus specificity in physical and biological assays. Removal of a non-viral fusion component typically improves the specificity and signal intensity for WN-rEIII.

In certain embodiments of the invention, methods for screening for a flavivirus in a subject include a) contacting a sample from the subject with a composition comprising a flavivirus domain III polypeptide under conditions that permit formation of specific immunocomplex between any antibody in the sample and the domain III polypeptide; and b) detecting whether a specific immunocomplex is formed. A domain III polypeptide refers to a polypeptide including the amino acids that define a structural element of a flavivirus envelope protein, for example amino acid sequences 292 to 402 of SEQ ID

NO:3 or homologous sequences from other flavivirus. Homologous domain III sequences from other flavivirus typically have an identity of at least 70, 75, 80, 85, 90, or 95 percent to the amino acid sequence 292-402 set forth in SEQ ID NO:3. Additionally, a specific immunocomplex refers to a complex between a polypeptide containing an epitope recognized by an antibody and the antibody that recognizes the epitope where the complex can be detected and distinguish above any non-specific or background interactions. The domain III polypeptide may be a dengue fever virus domain III polypeptide, yellow fever virus domain III polypeptide, West Nile virus domain III polypeptide, St. Louis encephalitis virus domain III polypeptide, Murray valley encephalitis virus domain III polypeptide or a combination or variant thereof. In particular embodiments the domain III polypeptide is a West Nile virus domain III polypeptide or a variant thereof. The domain III polypeptide may include 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60. 65. 70, 75, 80, 85, 90, or 94 contiguous amino acids of a flavivirus domain III polypeptide or a variant thereof. It is contemplated that 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more carboxy and/or amino terminal amino acids flanking domain III may also be included in a domain III polypeptide. A domain III polypeptide may include the amino acids 292-402 as set forth in SEQ ID NO:3, the amino acids 1-111 as set forth in SEQ ID NO:21, or variants thereof. Some embodiments of the invention further comprises at least a second domain III polypeptide. A second domain III polypeptide may be selected from SEQ ID NO:3-21. The domain III polypeptide may be prepared by isolating a recombinant or non-recombinant domain III polypeptide. The domain III polypeptide may be denatured or non-denatured. In particular embodiments the domain III polypeptide is prepared by isolating a recombinant domain III polypeptide fusion protein. In certain embodiments, a recombinant domain III polypeptide may be cleaved by an appropriate protease to separate the domain III polypeptide from its viral or non-viral fusion partner (*e.g.*, GST or MBP). A domain III polypeptide may be obtained from a bacteria comprising an expression vector encoding the domain III polypeptide or domain III polypeptide fusion protein. The domain III polypeptide or fusion protein may be obtained from a mammalian or insect cell comprising an expression vector encoding the domain III polypeptide or fusion protein.

In certain embodiments it is contemplated a domain III polypeptide may be used in conjunction with 1, 2, 3, 4, 5, 6, or more additional antigens derived the same or other members of the flavivirus family.

In various embodiments, samples may be derived from a variety of subjects infected with or suspected to be infected with a flavivirus, including WN. The subjects include, but are not limited to an animal, a bird, a human, a mosquito, a tick or other host organism for a flavivirus, in particular a West Nile virus.

The step of determining whether an immunocomplex is formed may be accomplished by a number of ways well known to those of ordinary skill in the art. The immunocomplex may be detected by ELISA, Western blotting, or peptide array. In other embodiments, an immunocomplex is detected using anti-antibody secondary reagents that refers to an agent that specifically bind an antibody. Compounds of the invention may be labeled with a detecting agent, which may be colorimetric, enzymatic, radioactive, chromatographic or fluorescent. The antigen may be affixed to a solid non-reactive support, which refers to a compound that will not react with antigens of the invention or antibodies in a any sample. The support may be a plate or assay dish, and be made of any non-reactive material, including, glass, plastic, silicon or the like. An antibody may include, but is not limited to an IgA or an IgM antibody.

Various embodiments include methods of identifying a flavivirus in a subject comprising a) contacting a sample from the subject with a composition comprising at least one flavivirus domain III polypeptide under conditions that permit formation of specific immunocomplex between any antibody in the sample and the domain III polypeptide; and b) detecting whether a specific immunocomplex is formed.

Certain embodiments of the invention include compositions for testing a sample for flavivirus comprising an isolated flavivirus domain III polypeptide. In particular embodiments, the flavivirus domain III polypeptide is a West Nile virus domain III polypeptide or variants thereof. The West Nile virus domain III polypeptide may be derived from West Nile strains USA99b, ETH76, 385-99, AUS60, ISR53, ISR52, MAD78, IND80 or a variant thereof, which may be obtained through the Arbovirus Reference Collection at the University of Texas Medical Branch at Galveston or similar depositories including the American Type Culture Collection. The composition may

include a flavivirus domain III polypeptide, which may comprise 10, 11, 12, 13, 14, 15, 16, 17, 18 , 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, or more, as well as values in between, consecutive amino acids of the domain III polypeptide or variants thereof. In particular embodiments, the composition may comprise the amino acid sequence as set forth in one or more of SEQ ID NO:3-21. The domain III polypeptide may be operatively linked to a substrate such as a plate, a microtiter plate, a bead, or a microarray.

Embodiments of the invention also include compositions for testing a sample for West Nile virus comprising an isolated flavivirus or West Nile virus domain III polypeptide as described above and incorporated here by reference.

Embodiments of the invention also include kits comprising any of the components of the invention described above, in a suitable container means. Kits may include one or more flavivirus or West Nile virus E protein domain III antigens. In still further embodiments, antigens are from the same or different strains. Such antigens may be in the same or in separate compositions. Kits may further include non-reactive supports in which antigens of the invention are affixed or attached. Kits may also include secondary antibody reagents. Antigens or antibodies in the kits may be labeled. Labels may be colorimetric, enzymatic, radioactive, or fluorescent. The domain III polypeptide may be a dengue fever virus domain III polypeptide, yellow fever virus domain III polypeptide, West Nile virus domain III polypeptide, St. Louis encephalitis virus domain III polypeptide, Murray valley encephalitis virus domain III polypeptide or a combination thereof. In particular embodiments, the domain III polypeptide is a West Nile virus domain III polypeptide. A kit may include compositions for screening for West Nile virus antibodies in a subject comprising: a) an assay plate comprising a multiplicity of microtiter wells comprising a composition comprising at least one domain III polypeptide capable of binding a flavivirus antibody in the sample that can specifically bind to at least one domain III polypeptide; and b) a container means comprising a labeled secondary antibody having specific binding affinity for a flavivirus antibody in the sample that can specifically bind to at least one domain III polypeptide.

Embodiments of the invention also include methods of screening for flavivirus in a subject comprising: a) contacting a sample from the subject with a composition from

the kit under binding conditions; and, b) detecting whether a specific immunocomplex is formed between an antibody and the at least one domain III polypeptide.

Various embodiments of the invention include vaccine compositions comprising a flavivirus or West Nile domain III polypeptide as described herein. The vaccine composition may further comprise an adjuvant(s) and an excipient(s) known in the art.

Other embodiments of the invention include an antibody or antibodies that selectively bind to an epitope in a domain III of a flavivirus or West Nile virus envelope protein. The epitope may be present in a West Nile domain III polypeptide or a variant thereof.

It is contemplated that any embodiment of a method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 illustrates an exemplary amino acid alignment of E protein domain IIIs from various flaviviruses.

FIG. 2 illustrates a two-dimensional schematic of the topology and structure of a flavivirus E protein.

FIG. 3 illustrates the binding of rabbit antiserum raised against WN recombinant domain III antigen to flavivirus envelope proteins in western blot assays with whole virus antigens of (1) WN, (2) JE, (3) SLE, and (4) MVE viruses.

FIG. 4 illustrates Western blot analysis or WN domain III specific monoclonal antibodies 5H10, 3A3, 7H2, 5C5, 3D9, and a polyclonal antiserum to WN domain III.

FIG. 5 illustrates the results of an exemplary PRNT assay showing the neutralization activity of rabbit anti-domain III sera.

FIG. 6 illustrates an E protein domain III amino acid sequence variations for ten West Nile virus strains, and representative JE (Genbank accession U21057), SLE (M16614) and MVE (M24220) viruses. Dots (.) indicate conservation with the USA99b sequence. Residues associated with escape from neutralization by Mabs or anti-domain III serum for WN virus strains are shaded.

FIG. 7 illustrates the binding of selected anti-flavivirus mouse immune asicitic fluids in an indirect ELISA protocol utilizing whole-virus JE serocomplex antigens (WN, JE, SLE, or MVE viruses) or recombinant WN E protein domain III. Error bars 1 standard deviation from the mean.

FIG. 8 illustrates the binding of selected anti-flavivirus mouse immune asicitic fluids in an indirect ELISA protocol utilizing whole-virus JE serocomplex antigens (WN, JE, SLE, or MVE viruses) or recombinant WN E protein domain III cleaved from a GST fusion protein.

FIG. 9 illustrates the binding of selected anti-flavivirus mouse immune asicitic fluids in an indirect ELISA protocol utilizing WN rEIII cleaved from an MBP fusion protein, MBP WN rEIII fusion protein at 35 ng/well, and MBP WN rEIII fusion protein at 17.5 ng/well.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Various embodiments of the invention include compositions and methods related to flavivirus or West Nile virus E protein domain III (EIII) or recombinant EIII (rEIII) as an antigen for specific diagnosis or detection of WN virus. The flavivirus envelope (E) protein is the major virion surface protein. It plays an important role in virus attachment

and entry into host cells, and is also an important target for virus neutralizing antibodies (Sanchez and Ruiz, 1996; Mandl *et al.*, 2000; Crill and Roehrig, 2001). The inventors described the identification of residues associated with the neutralization of lineage I WN virus strain 385-99 (isolated in New York City in 1999) by monoclonal antibodies (MAbs) that bound to EIII, the putative receptor-binding domain, of the E protein.

Using these EIII-reactive MAbs and a polyclonal serum generated against a recombinant, bacterially-expressed WN virus rEIII fragment, the antigenic relationships between WN virus strains representative of genetic lineages I and II and identified residues in domain III that constitute subtype specific epitopes have been investigated.

The present invention includes compositions and methods for the detection or diagnosis of a flavivirus, including compositions and methods for distinguishing between different flaviviruses. In particular embodiments, the flavivirus being detected is the West Nile virus. Recombinant West Nile virus E protein domain III (rEIII) can be expressed in *E. coli* as a fusion protein to produce a soluble protein that can easily be purified. Rabbit antisera raised against rEIII shows virus specificity in physical and biological assays. Removal of the fusion component improves specificity and signal intensity for rEIII.

I. FLAVIVIRUS

West Nile virus is a member of the genus *Flavivirus*. *Flavivirus* is a member of the *Flaviviridae* family and includes the viral subgroups of Yellow Fever virus group, Tick-borne encephalitis virus group, Rio Bravo Group, Japanese encephalitis Group, Tyuleniy Group, Ntaya Group, Uganda S Group, Dengue Group, and Modoc Group. Members of the *Flavivirus* genus may produce a wide variety of disease states, such as fever, arthralgia, rash, hemorrhagic fever, and/or encephalitis. The outcome of infection is influenced by both the virus and host-specific factors, such as age, sex, genetic susceptibility, and/or pre-exposure to the same or a related agent. Some of the various diseases associated with members of the genus *Flavivirus* are Yellow Fever; Dengue Fever; and West Nile, Japanese, and St. Louis Encephalitis.

Serological comparisons of West Nile virus strains have distinguished four major antigenic subtypes: a group of strains from Africa; strains from Europe and some Asian

strains; strains from India; and strains of Kunjin virus from Australasia (Doherty *et al.*, 1968; Hammam *et al.*, 1966; Blackburn *et al.*, 1987; Calisher *et al.*, 1989; Morvan *et al.*, 1990). Subsequently, analyses of nucleotide sequences identified two major genetic lineages, designated I and II, which included some subtypes and which correlated well with the antigenic groupings. Genetic lineage I included European and some African strains, Kunjin virus strains, and Indian strains; lineage II comprised only African strains (Lanctiotti *et al.*, 1999; Jia *et al.*, 1999; Scherret *et al.*, 2001).

Virions of the *Flaviviridae* generally contain one molecule of a linear positive-sense single stranded RNA genome of approximately 10,000-11,000 nucleotides that replicates in the cytoplasm of an infected cell. Typically the 5' end of the genome has a cap and the 3' end that may or may not have a poly (A) tract. *Flavivirus* are usually transmitted by a vector such as an insect, in many cases the insect is a mosquito.

The viral genome of the *Flavivirus* genus is translated as a single polypeptide and is subsequently cleaved into mature proteins. The proteins encoded by the virus typically consist of structural and non-structural proteins. Generally, there are three structural proteins that typically include the envelope protein (E protein)(amino acids 275-787 of GenBank accession number NP_041724, incorporated herein by reference and SEQ ID NO:2), the core or capsid protein (C)(amino acids 1-92 of GenBank accession number NP_041724), and the pre-membrane protein (preM)(amino acids 105-223 of GenBank accession number NP_041724)(Yamshchikov *et al.*, 2001, incorporated herein by reference). The envelope protein is approximately 496 amino acids with an approximate molecular weight of 50 kDa and is often glycosylated. The envelop protein typically contains twelve conserved cysteine residues which form six disulfide bridges. The core protein is approximately 13 kDa and is rich in arginine and lysine residues. The pre-membrane protein is approximately 10 kDa and is cleaved during or after release of the virus from infected cells. A cleavage product of the prM protein remains associated with the virion and is approximately 8 kDa and is termed the membrane protein (M). Typically, it is the carboxy terminus of prM that remains associated with the virus particle as the M protein.

The flavivirus E protein is a dimer positioned parallel to virus surface. It includes three domains I- Central domain (EI), II- Dimerization domain (EII), III-

Immunogenic/Receptor binding domain (EIII) (FIG. 2). The amino acid sequence of an exemplary West Nile virus E protein Domain III is set forth in SEQ ID NO:3. An amino acid alignment of various flavivirus EIIIs is presented in FIG. 1. The E protein domain III is approximately 10.5 kDa with a single disulfide bridge. The E protein domain III has an Ig-like fold, which is a β-barrel "type" configuration with no α-helices. Some flavivirus E protein domain IIIs contain a RGD integrin-binding motif.

Various members of the *Flaviviridae* family are available through the American Type Culture Collection (Manassas Va.) under the following ATCC numbers: Dengue type 1 (VR-71), Ilheus (VR-73), Japanese encephalitis (VR-74), Murray valley encephalitis (VR-77), Ntaya (VR-78), St Louis encephalitis (VR-80), Uganda S (VR-81), West Nile (VR-82), Zika (VR-84), Dengue type 4 (VR-217), Dengue type 2 (VR-222), Japanese encephalitis (VR-343), Dengue type 1 (VR-344), Dengue type 2 (VR-345), Edge hill (VR-377), Entebbe bat (VR-378), Kokobera (VR-379), Stratford (VR-380), Tembusu (VR-381), Dakar bat (VR-382), Ntaya (VR-78), Banzi (VR-414), Modoc (VR-415), Rio Bravo virus (VR-416), Cowbone ridge (VR-417), Bukalasa (VR-418), Montana myotis leukoencephalitis (VR-537), Bussuquara (VR-557), Sepik (VR-906), Cowbone ridge (VR-1253), Dengue type 2 (VR-1255), Dengue type 3 (VR-1256), Dengue type 4 (VR-1257), Ilheus (VR-1258), Rio Bravo virus (VR-1263), St. Louis encephalitis (VR-1265), West Nile (VR-1267), Dengue type 4 (VR-1490), West Nile (VR-1507), and West Nile (VR-1510), each of which is incorporated herein by reference.

II. PROTEINACEOUS COMPOSITIONS

In various embodiments of the invention *flavivirus* or West Nile virus polypeptides or proteins may be comprised in various proteinaceous compositions. These proteinaceous composition may be used in the detection of *flavivirus* members, vaccination against *flavivirus* members, as well as other methods and compositions described herein.

A. Proteinaceous Compositions

In certain embodiments, the present invention concerns novel compositions comprising at least one proteinaceous molecule, such as a r-EIII polypeptide (antigen) alone or in combination with other flavivirus E proteins, E protein domain IIIs or

fragments thereof. As used herein, a "proteinaceous molecule," "proteinaceous composition," "proteinaceous compound," "proteinaceous chain" or "proteinaceous material" generally refers, but is not limited to, a protein of greater than about 200 amino acids or the full length endogenous sequence translated from a gene; a polypeptide of greater than about 100 amino acids; and/or a peptide of from about 3 to about 100 amino acids. All the "proteinaceous" terms described above may be used interchangeably herein. The term "antigen" refers to any substance or material that is specifically recognized by an antibody or T cell receptor, and it is used interchangeably with the term "epitope," which refers to an antigenic determinant. Thus, it is contemplated that the antigens of the invention may be truncations or only portions of a full-length polypeptide. For example, a "r-EIII antigen" refers to a peptide or polypeptide containing contiguous amino acids of E protein domain III, including at least one E protein domain III epitope, but it may be fewer than a full-length amino acid sequence. Thus, a E protein domain III antigen may include a region of contiguous amino acids of any of SEQ ID NO:3-21.

SEQ ID NO:2 corresponds to protein accession number NP_041724, which is the sequence for a West Nile virus. SEQ ID NO:3 corresponds to amino acids 291-787 of SEQ ID NO:2 , which is a full-length processed E protein domain III polypeptide sequence. Immunogenic regions of flavivirus envelope proteins have been described, and the present invention includes antigens that include one or more such regions.

In certain embodiments, a proteinaceous molecule comprising an West Nile virus domain III antigen may comprise, be at least, or be at most 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1100, 1200, 1300, 1400, 1500, 1750, 2000, 2250, 2500 or greater contiguous amino acid residues, and any range derivable therein of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3-21.

As used herein, an "amino molecule" refers to any amino acid, amino acid derivative or amino acid mimic as would be known to one of ordinary skill in the art. In certain embodiments, the residues of the proteinaceous molecule are sequential, without any non-amino molecule interrupting the sequence of amino molecule residues. In other embodiments, the sequence may comprise one or more non-amino molecule moieties. In particular embodiments, the sequence of residues of the proteinaceous molecule may be interrupted by one or more non-amino molecule moieties.

Encompassed by certain embodiments of the present invention are peptides, such as, for example, a peptide comprising all or part of a flavivirus envelope antigen (including at least one epitope) of any subtype or clade. Peptides of the invention may comprise, be at least, or be at most 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 contiguous amino acids, including all or part of any of SEQ ID NO:2-21.

Accordingly, the term "proteinaceous composition" encompasses amino molecule sequences comprising at least one of the 20 common amino acids in naturally synthesized proteins, or at least one modified or unusual amino acid, including but not limited to those shown on Table 1 below.

TABLE 1
Modified and Unusual Amino Acids

Abbr.	Amino Acid	Abbr.	Amino Acid
Aad	2-Aminoadipic acid	EtAsn	N-Ethylasparagine
Baad	3- Aminoadipic acid	Hyl	Hydroxylysine
Bala	β-alanine, β-Amino-propionic acid	AHyl	allo-Hydroxylysine
Abu	2-Aminobutyric acid	3Hyp	3-Hydroxyproline
4Abu	4- Aminobutyric acid, piperidinic acid	4Hyp	4-Hydroxyproline
Acp	6-Aminocaproic acid	Ide	Isodesmosine
Ahe	2-Aminoheptanoic acid	Alle	allo-Isoleucine

TABLE 1 Modified and Unusual Amino Acids			
Abbr.	Amino Acid	Abbr.	Amino Acid
Aib	2-Aminoisobutyric acid	MeGly	N-Methylglycine, sarcosine
Baib	3-Aminoisobutyric acid	MeIle	N-Methylisoleucine
Apm	2-Aminopimelic acid	MeLys	6-N-Methyllysine
Dbu	2,4-Diaminobutyric acid	MeVal	N-Methylvaline
Des	Desmosine	Nva	Norvaline
Dpm	2,2'-Diaminopimelic acid	Nle	Norleucine
Dpr	2,3-Diaminopropionic acid	Orn	Ornithine
EtGly	N-Ethylglycine		

In certain embodiments the proteinaceous composition comprises at least one protein, polypeptide or peptide. In further embodiments the proteinaceous composition comprises a biocompatible protein, polypeptide or peptide. As used herein, the term "biocompatible" refers to a substance which produces no significant untoward effects when applied to, or administered to, a given organism according to the methods and amounts described herein. Such untoward or undesirable effects are those such as significant toxicity or adverse immunological reactions. In preferred embodiments, biocompatible protein, polypeptide or peptide containing compositions will generally be mammalian proteins or peptides or synthetic proteins or peptides each essentially free from toxins, pathogens and harmful immunogens.

Proteinaceous compositions may be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, the isolation of proteinaceous compounds from natural sources, or the chemical synthesis of proteinaceous materials. The nucleotide and protein, polypeptide and peptide sequences for various genes have been previously disclosed, and may be found at computerized databases known to those of ordinary skill in the art. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases (http://www.ncbi.nlm.nih.gov/). The coding regions for

these known genes may be amplified and/or expressed using the techniques disclosed herein or as would be know to those of ordinary skill in the art. Alternatively, various commercial preparations of proteins, polypeptides and peptides are known to those of skill in the art.

In certain embodiments a proteinaceous compound may be purified. Generally, "purified" will refer to a specific or protein, polypeptide, or peptide composition that has been subjected to fractionation to remove various other proteins, polypeptides, or peptides, and which composition substantially retains its activity, as may be assessed, for example, by the protein assays, as would be known to one of ordinary skill in the art for the specific or desired protein, polypeptide or peptide. In still further embodiments, a proteinaceous compound may be purified to allow it to retain its native or non-denatured conformation. Such compounds may be recombinantly derived or they may be purified from endogenous sources.

In certain embodiments, the proteinaceous composition may comprise at least one antigen of E protein domain III that is recognized by an antibody. As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting.

The term "antibody" is also used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, $F(ab')_2$, single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, *e.g.*, Harlow *et al.*, 1988; incorporated herein by reference).

It is contemplated that virtually any protein, polypeptide or peptide containing component may be used in the compositions and methods disclosed herein. However, it is preferred that the proteinaceous material is biocompatible. In certain embodiments, it is envisioned that the formation of a more viscous composition will be advantageous in that it will allow the composition to be more precisely or easily applied to the tissue and to be maintained in contact with the tissue throughout the procedure. In such cases, the

use of a peptide composition, or more preferably, a polypeptide or protein composition, is contemplated. Ranges of viscosity include, but are not limited to, about 40 to about 100 poise. In certain aspects, a viscosity of about 80 to about 100 poise is preferred.

1. Variants of Flavivirus Envelope Protein Domain III Antigens

Amino acid sequence variants of the polypeptides of the present invention can be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the native protein that are not essential for function or immunogenic activity, and are exemplified by the variants lacking a transmembrane sequence described above. Another common type of deletion variant is one lacking secretory signal sequences or signal sequences directing a protein to bind to a particular part of a cell. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of an immunoreactive epitope or simply a single residue. Terminal additions, called fusion proteins, are discussed below.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, such as stability against proteolytic cleavage, without the loss of other functions or properties. Substitutions of this kind preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine or histidine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids (see Table 2, below).

It also will be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences,

and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

TABLE 2

Codon Table

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies. Since it is the interactive capacity and

nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and in its underlying DNA coding sequence, and nevertheless produce a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity, as discussed below. Table 2 shows the codons that encode particular amino acids.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still produce a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take into

consideration the various foregoing characteristics are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See *e.g.*, Johnson (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. These principles may be used, in conjunction with the principles outline above, to engineer second generation molecules having many of the properties of flavivirus envelope protein domain III antigens, but with altered and even improved characteristics.

2. Fusion Proteins

A specialized kind of insertional variant is the fusion protein. This molecule generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide. For example, fusions typically employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of a region to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful fusions include linking of functional domains, such as active sites from enzymes such as a hydrolase, glycosylation domains, cellular targeting signals or transmembrane regions.

3. Protein Purification

It is desirable to purify flavivirus envelope protein domain III antigens or variants thereof. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Certain embodiments of the invention are directed at preserving the conformation of flavivirus envelope protein domain III antigens as much as possible so that they are substantially non-denatured.

Antigens of the invention may be purified using gentle, non-denaturing detergents, which include, but are not limited to, NP40 and digitonin. Infected or

transfected host cells may be solubilized using a gentle detergent. The following conditions are considered "substantially denaturing" or "denaturing": 10 mM CHAPS, 0.5% SDS, >2% deoxycholate, or 2.0% octylglucoside. Antigens prepared under such conditions would not be considered "non-denatured antigens." Preparations of substantially non-denatured antigens of the invention may be accomplished using techniques described in U.S. Patents 6,074,646 and 5,587,285, which are hereby incorporated by reference herein.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

4. Antibodies

The present invention concerns the detection of flavivirus or West Nile virus antibodies using flavivirus or West Nile virus antigens. As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting. As described earlier, an antigen may include one or more epitopes and an antigen refers to any part of a polypeptide that contains at least one epitope.

The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (*See, e.g.*, Harlow and Lane, "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

In addition to polypeptides, antigens of the invention may be peptides corresponding to one or more antigenic determinants of the flavivirus envelope protein domain III polypeptides of the present invention. Thus, it is contemplated that detection of an flavivirus or West Nile virus antibody may be accomplished with an flavivirus envelope protein domain III antigen that is a peptide or polypeptide.

Such peptides should generally be at least five or six amino acid residues in length and will preferably be about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or about 30 amino acid residues in length, and may contain up to about 35-111 residues. For example, these peptides may comprise a WN EIII antigen sequence, such as 5, 6, 7, 8, 9,

10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 110 or more contiguous amino acids from any of SEQ ID NO:3-21. Synthetic peptides will generally be about 35 residues long, which is the approximate upper length limit of automated peptide synthesis machines, such as those available from Applied Biosystems (Foster City, CA). Longer peptides also may be prepared, *e.g.*, by recombinant means.

U.S. Patent 4,554,101, incorporated herein by reference, teaches the identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed, one of skill in the art would be able to identify epitopes and/or antigens from within an amino acid sequence such as a flavivirus or West Nile virus sequence disclosed herein in as SEQ ID NO:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21.

Numerous scientific publications have also been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou and Fasman, 1974a, b; 1978a, b; 1979). Any of these may be used, if desired, to supplement the teachings of Hopp in U.S. Patent 4,554,101.

Moreover, computer programs are currently available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson and Wolf, 1988; Wolf *et al.*, 1988), the program PepPlot® (Brutlag *et al.*, 1990; Weinberger *et al.*, 1985), and other new programs for protein tertiary structure prediction (Fetrow and Bryant, 1993). Another commercially available software program capable of carrying out such analyses is MacVector (IBI, New Haven, CT).

In further embodiments, major antigenic determinants of flavivirus or West Nile envelope protein domain III polypeptide may be identified by an empirical approach in which portions of the gene encoding a flavivirus or West Nile envelope protein are expressed in a recombinant host, and the resulting proteins tested for their ability to elicit an immune response. Alternatively all or part of flavivirus envelope proteins from different subtypes or clades may be tested. A range of peptides lacking successively longer fragments of the C-terminus of the protein can be assayed as long as the peptides are prepared to retain their structure as it would be in a native polypeptide. The immunoactivity of each of these peptides is determined to identify those fragments or

domains of the polypeptide that are immunodominant. Further studies in which only a small number of amino acids are removed at each iteration then allows the location of the antigenic determinants of the polypeptide to be more precisely determined.

Once one or more such analyses are completed, polypeptides are prepared that contain at least the essential features of one or more antigenic determinants. The peptides are then employed in the generation of antisera against the polypeptide. Minigenes or gene fusions encoding these determinants also can be constructed and inserted into expression vectors by standard methods, for example, using PCR™ cloning methodology.

5. Immunodetection Methods

As discussed, in some embodiments, the present invention concerns immunodetection methods for binding, purifying, removing, quantifying and/or otherwise detecting flavivirus antibodies in a sample, particularly West Nile virus antibodies, using EIII antigens. The samples may be any biological fluid or tissue from a patient. The sample may be placed on a non-reactive surface such as a plate, slide, tube, or other structure that facilitates in any way the screening of the sample for flavivirus antibodies. While samples may be individually screened, large numbers of samples may be screened, such as for detecting contamination in blood bank samples.

Immunodetection methods include enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoradiometric assay, fluoroimmunoassay, chemiluminescent assay, bioluminescent assay, and Western blot, though several others are well known to those of ordinary skill. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Doolittle *et al.*, 1999; Gulbis *et al.*, 1993; De Jager *et al.*, 1993; and Nakamura *et al.*, 1987, each incorporated herein by reference.

In general, the immunobinding methods include obtaining a sample suspected of containing a flavivirus, in particular a West Nile virus antibody with a composition comprising a flavivirus or West Nile EIII antigen in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes.

These methods include methods for purifying a antibody from organelle, cell, tissue or organism's samples. In these instances, the antigen removes the antibody

component from a sample. The antigen will preferably be linked to a solid support, such as in the form of a column matrix, and the sample suspected of containing the antibody will be applied to the immobilized antigen. The unwanted components will be washed from the column, leaving the antibody immunocomplexed to the immobilized antigen to be eluted. Alternatively, sandwich versions of this assay may be employed.

The immunobinding methods also include methods for detecting and quantifying the amount of an antibody component in a sample and the detection and quantification of any immune complexes formed during the binding process. Here, one would obtain a sample suspected of containing an antibody and contact the sample with an antigen, and then detect and quantify the amount of immune complexes formed under the specific conditions.

In terms of antigen detection, the biological sample analyzed may be any sample that is suspected of containing an antibody, such as, for example, a tissue section or specimen, a homogenized tissue extract, a cell, an organelle, separated and/or purified forms of any of the above antibody-containing compositions, or even any biological fluid that comes into contact with the cell or tissue, including blood and/or serum..

Contacting the chosen biological sample with the antigen under effective conditions and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the antigen composition to the sample and incubating the mixture for a period of time long enough for any antibodies present to form immune complexes with, *i.e.*, to bind to, antigens . After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any of those radioactive, fluorescent, biological and enzymatic tags. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional

advantages through the use of a secondary binding ligand such as a second antibody and/or a biotin/avidin ligand binding arrangement, as is known in the art.

The antigen employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined. Alternatively, the first antigen that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antigen. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under effective conditions and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under effective conditions and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is desired.

a. ELISAs

As detailed above, immunoassays, in their most simple and/or direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and/or radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and/or western blotting, dot blotting, FACS analyses, and/or the like may also be used.

Turning first to immunoassays, in their most simple and direct sense, preferred immunoassays of the invention include the various types of enzyme linked immunosorbent assays (ELISAs) known to the art. However, it will be readily appreciated that the utility of the EIII preparations described herein are not limited to such assays, and that other useful embodiments include RIAs and other non-enzyme linked antibody binding assays or procedures.

In some embodiments of the ELISA assay, flavivirus or West Nile virus envelope proteins or appropriate peptides incorporating EIII antigen sequences are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, one will desire to bind or coat a nonspecific protein such as bovine serum albumin (BSA), casein, solutions of milk powder, gelatin, PVP, superblock, or horse albumin onto the well that is known to be antigenically neutral with regard to the test antisera. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface. Following an appropriate coating period (for example, 3 hours), the coated wells will be blocked with a suitable protein, such as bovine serum albumin (BSA), casein, solutions of milk powder, gelatin, PVP, superblock, or horse albumin, and rinsed several times (e.g., 4 or 5 times) with a suitable buffer, such as PBS. The wells of the plates may then be allowed to dry, or may instead be used while they are still wet.

After binding of antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the antisera or clinical or biological extract to be tested in a manner conducive to immune complex (antigen/antibody) formation. Such conditions preferably include diluting the antisera with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background. The layered antisera is then allowed to incubate for from 1 to 4 hours, at temperatures preferably on the order of 20° to 25°C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound antigen, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for the first. Of course, in that the test sample will typically be of human origin, the second antibody will preferably be an antibody having specificity in general for human IgG, IgM or IgA. To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antisera-bound surface with a urease, alkaline phosphatase, or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and H_2O_2, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

In each of the microtiter wells will be placed about 10 µl of the test patient sample along with about 90 µl of reaction buffer (*e.g.*, PBS with about 1% digitonin or other mild protein solubilizing agent). Control wells of the ELISA plate will include normal sera (human sera without flavivirus antibody), anti-flavivirus antibody collected from subjects.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating and binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described below.

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test

antisera. These include bovine serum albumin (BSA), casein or solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, and a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or a third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and/or antibodies with solutions such as BSA, bovine gamma globulin (BGG) or phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature or for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours or so, at temperatures preferably on the order of 25°C to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. An example of a washing procedure includes washing with a solution such as PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. This may be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact or incubate the first and second immune complex with

a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea, or bromocresol purple, or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid (ABTS), or H_2O_2, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generated, *e.g.*, using a visible spectra spectrophotometer.

b. Assay Plates

In some embodiments, the wells of the assay plates may first be coated with an anti-EIII and/or anti-WN-EIII antibody. This would immobilize EIII antigen to the plastic in the presence of a mild solubilizing buffer, such as from about 0.1% to about 10% digitonin (particularly about 1% digitonin). Such an approach is particularly efficacious in preparing assay plates with wells made of plastic.

The assay plates in other embodiments of the invention comprise a multiplicity of microtiter wells, and in some embodiments, polystyrene microtiter wells. These wells would be coated with about 500 ng/well of the rEIII, or WN-rEIII antigen.

c. Immunohistochemistry

The antigens of the present invention may also be used in conjunction with both fresh-frozen and/or paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). Flavivirus and West Nile virus antibodies may be identified in this manner. The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and/or is well known to those of skill in the art (Brown *et al.*, 1990; Abbondanzo *et al.*, 1990; Allred *et al.*, 1990).

Briefly, frozen-sections may be prepared by rehydrating 50 mg of frozen "pulverized" tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and/or pelleting again by

centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and/or removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and/or cutting 25-50 serial sections.

Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and/or embedding the block in paraffin; and/or cutting up to 50 serial permanent sections.

III. NUCLEIC ACID MOLECULES

In some embodiments, the present invention concerns E protein domain III envelope antigens prepared from genomic or recombinant nucleic acids. Some of the teachings herein pertain to the construction, manipulation, and use of nucleic acids to produce a recombinant E protein domain III envelope antigen.

A. Polynucleotides Encoding E protein domain III Envelope Antigens

The present invention concerns polynucleotides, isolatable from cells, that are free from total genomic DNA and that are capable of expressing all or part of a protein or polypeptide. The polynucleotide may encode a peptide or polypeptide containing all or part of an E protein domain III amino acid sequence or may encode a peptide or polypeptide having an E protein domain III antigen sequence. Recombinant proteins can be purified from expressing cells to yield denatured or non-denatured proteins or peptides.

As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding a polypeptide refers to a DNA segment that contains wild-type, polymorphic, or mutant polypeptide-coding sequences yet is isolated away from, or purified free from, total mammalian or human genomic DNA. Included within the term "DNA segment" are recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

As used in this application, the term "E protein domain III (EIII) polynucleotide" refers to an E protein domain III polypeptide-encoding nucleic acid molecule that has been

isolated free of total genomic nucleic acid. Therefore, a "polynucleotide encoding an E protein domain III antigen" refers to a DNA segment that contains all or part of E protein domain III polypeptide-coding sequences isolated away from, or purified free from, total viral genomic nucleic acid.

It also is contemplated that a particular polypeptide from a given species or strain may be represented by natural variants that have slightly different nucleic acid sequences but, nonetheless, encode the same protein (see above).

Similarly, a polynucleotide comprising an isolated or purified gene refers to a DNA segment including, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide, or peptide-encoding unit. As will be understood by those in the art, this functional term includes genomic sequences, cDNA sequences, RNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins, and mutants. A nucleic acid encoding all or part of a native or modified polypeptide may contain a contiguous nucleic acid sequence encoding all or a portion of such a polypeptide of the following lengths: about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040, 1050, 1060, 1070, 1080, 1090, 1095, 1100, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 9000, 10000, or more nucleotides, nucleosides, or base pairs, which may be contiguous nucleotides encoding any length of contiguous amino acids of SEQ ID NO:2, or any of SEQ ID NO:3-21.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode an EIII antigen polypeptide or peptide, such as all or part of EIII, which includes within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially

corresponding to a native polypeptide. Thus, an isolated DNA segment or vector containing a DNA segment may encode, for example, a EIII antigen that is capable of binding to an flavivirus antibody. The term "recombinant" may be used in conjunction with a polypeptide or the name of a specific polypeptide, and this generally refers to a polypeptide produced from a nucleic acid molecule that has been manipulated *in vitro* or that is the replicated product of such a molecule.

Encompassed by certain embodiments of the present invention are DNA segments encoding relatively small peptides, such as, for example, a peptide comprising all or part of an EIII envelope antigen (including at least one epitope) of any subtype or clade.

In other embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode a polypeptide or peptide that includes within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially corresponding to the polypeptide.

The nucleic acid segments used in the present invention, regardless of the length of the coding sequence itself, may be combined with other nucleic acid sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

It is contemplated that the nucleic acid constructs of the present invention may encode full-length polypeptide from any source or encode a truncated version of the polypeptide, for example a truncated E protein domain III polypeptide, such that the transcript of the coding region represents the truncated version. The truncated transcript may then be translated into a truncated protein. Alternatively, a nucleic acid sequence may encode a full-length polypeptide sequence with additional heterologous coding sequences, for example to allow for purification of the polypeptide, transport, secretion, post-translational modification, or for therapeutic benefits such as targeting or efficacy. As discussed above, a tag or other heterologous polypeptide may be added to the modified polypeptide-encoding sequence, wherein "heterologous" refers to a polypeptide that is not the same as the modified polypeptide.

In a non-limiting example, one or more nucleic acid constructs may be prepared that include a contiguous stretch of nucleotides identical to or complementary to the a particular gene, such as a E protein gene of a particular subtype. A nucleic acid construct may be at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000 nucleotides in length, as well as constructs of greater size, up to and including chromosomal sizes (including all intermediate lengths and intermediate ranges), given the advent of nucleic acids constructs such as a yeast artificial chromosome are known to those of ordinary skill in the art. It will be readily understood that "intermediate lengths" and "intermediate ranges," as used herein, means any length or range including or between the quoted values (*i.e.*, all integers including and between such values).

The DNA segments used in the present invention encompass biologically functional equivalent modified polypeptides and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by human may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein, to reduce toxicity effects of the protein *in vivo* to a subject given the protein, or to increase the efficacy of any treatment involving the protein.

The sequence of a flavivirus EIII polypeptide will substantially correspond to a contiguous portion of that shown in amino acids 292-402 of SEQ ID NO:3 or any of SEQ ID NO:4-21 and have relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids shown in amino acids 292-402 of SEQ ID NO:3 or any of SEQ ID NO:4-21. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein to include an ability to bind or be recognized by a specific flavivirus antibody.

Accordingly, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:3-21 will be sequences that are "essentially as set forth in SEQ ID NO:3-21."

In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that include within their sequence a contiguous nucleic acid sequence from that shown in SEQ ID NO:1. This definition is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a contiguous portion of that shown in SEQ ID NO:1 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:1. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids. See Table 2 above, which lists the codons preferred for use in humans, with the codons listed in decreasing order of preference from left to right in the table (Wada *et al.*, 1990). Codon preferences for other organisms also are well known to those of skill in the art (Wada *et al.*, 1990, included herein in its entirety by reference).

The various probes and primers designed around the nucleotide sequences of the present invention may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, *etc.*, an algorithm defining all primers can be proposed:

n to n + y

where n is an integer from 1 to the last number of the sequence and y is the length of the primer minus one, where n + y does not exceed the last number of the sequence. Thus, for a 10-mer, the probes correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and so on. For a 15-mer, the probes correspond to bases 1 to 15, 2 to 16, 3 to 17 ... and so on. For a 20-mer, the probes correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and so on.

It also will be understood that this invention is not limited to the particular nucleic acid encoding amino acid sequences of SEQ ID NO:2, or any of SEQ ID NO:3-21.

Recombinant vectors and isolated DNA segments may therefore variously include the EIII antigen-coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that nevertheless include EIII antigen-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

The DNA segments of the present invention encompass biologically functional equivalent EIII antigen proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein.

1. Vectors

Native and modified polypeptides may be encoded by a nucleic acid molecule comprised in a vector. The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.*, YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques, which are described in Sambrook *et al.*, (1989) and Ausubel *et al.*, 1996, both incorporated herein by reference. In addition to encoding a modified polypeptide such as modified EIII, a vector may encode non-modified polypeptide sequences such as a tag or targeting molecule. Useful vectors encoding such fusion proteins include pIN vectors (Inouye *et al.*, 1985), vectors encoding a stretch of histidines, and pGEX vectors, for use in generating glutathione S-transferase (GST) soluble

fusion proteins for later purification and separation or cleavage. A targeting molecule is one that directs the modified polypeptide to a particular organ, tissue, cell, or other location in a subject's body.

The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

Vectors may include a "promoter," which is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′– methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patent 5,925,565 and 5,935,819, herein incorporated by reference).

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. (See Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997, incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.